

Quantification of thymol content in different extracts of *Zataria multiflora* by HPLC method

Azar Aghamohammadi¹, Mohammad Azadbakht^{1,2}, Seyed Jalal Hosseinimehr^{1,3*}

¹Traditional and Complementary Medicine Research Center, Mazandaran University of Medical Sciences, Sari, Iran

²Department of Pharmacognosy, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran

³Department of Radiopharmacy, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran

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Abstract

Zataria multiflora Boiss is used in traditional folk remedies as antiseptic, analgesic, carminative, anthelmintic medication. The main components of *Z. multiflora* are phenolic compounds such as thymol and carvacrol. The aim of this study was developed a simple and rapid method for determination of the thymol in different extracts of *Z. multiflora* by HPLC method. The dried aerial parts of *Z. multiflora* in the flowering stage was extracted with ethanol 34%, 42% and 70% for 48 hours. Several mobile phase systems were applied for development and separation of thymol and carvacrol that have close retention time in HPLC system. The peaks of thymol and carvacrol are successfully separated in acetonitrile-water-acetic acid mobile phase. Thymol and carvacrol were separated with a retention times 10.4 and 9.8 minutes respectively, in an isocratic solvent system with HPLC. Thymol content was 2.7 ± 0.06 , 3.7 ± 0.07 and 6.0 ± 0.11 mg/g, in ethanol at concentration of 34%, 42% and 70%, respectively. Thymol content in different hydroalcoholic extract of *Z. multiflora* is dependent to ethanol concentration in extraction solvent.

Keywords: *Z. multiflora*, thymol, carvacrol, HPLC

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Introduction

Lamiaceae family is one of the largest herbals' families that are growing in the world and has 200 genus and 2000-5000 species of aroma. *Zataria multiflora* Boiss. (ZM) (synonyms: *Zataria bracteata* Boiss.; *Zataria multiflora* var. *elatior* Boiss) is a thyme-like plant and a member of this family with multiple, thin, hard and forked stalks (1,2). ZM is containing thymol, carvacrol, zatrinal, oleanolic acid, betulic acid, rosmarinic acid and monoterpenoids such as sesquiterpenoids, p-cymene and

terpinene (2). The main components of ZM oil are phenolic compounds. The biological effects of *Z. multiflora* are mainly associated to its phenolic compounds, especially thymol and carvacrol (Fig. 1) (3). ZM has several biological and pharmacological properties including anti-fungal (4), antierythma (5), anti-nociceptive (6), anti oxidative stress (7,8), antimicrobial (2, 9) spasmolytic, anti-inflammatory (10), immunostimulant (11) pain-relieving (12) and radioprotective

* E-mail: sjhosseinim@yahoo.com

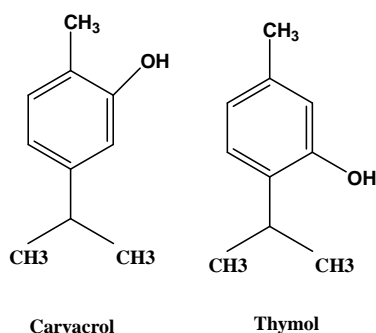


Figure 1 Chemical structures of phenolic compound (thymol and carvacrol)

effects (13). In Iranian traditional medicine, ZM is used for carminative properties and analgesic. It is used for treatment of Candidiasis vaginitis and some pharmaceutical forms of this plant, such as syrups, drops, soft capsules and vaginal creams are sold as treatments for various diseases (2). Several studies analyzed carvacrol and thymol as the main compounds in the essential oil of ZM. Dehkordi *et al.* collected ZM from five different areas of Iran and analyzed its oils. According to the gas chromatography/mass spectrometry (GC-MS) data, the main oil constituents remained similar between plants from different geographical regions, but their relative quantities differed among plants from different regions. Thymol was the most abundant compound among all constituents in all samples (4).

In spite of the extensive uses of ZM in medicine and most of the pharmaceutical dosage forms, phytochemical investigations of ZM have been frequently focused on the essential oil and there are not any studies to standardized ZM hydroalcoholic extract to their thymol content and separation of thymol and carvacrol peaks by HPLC method.

However, type of solvent is affected on thymol extraction from extract. This study was performed for quantification of the thymol in different extracts of ZM by HPLC method.

Materials and Methods

Plant material

The dried aerial parts of *Zataria multiflora* was collected from their major growing areas around Firozabad city of Fars province GO (28.8194258° N, 52.5518705° W), Iran at the full flowering stage (June and July 2012). It was confirmed by a senior botanist Prof. Mohammad Azadbakht at the Mazandaran University of Medical Science, Iran (Herbarium number: F-1-8-4-21). Five grams of the homogenous powder (1 mm in diameter mesh No. 18) with 50 ml hydroalcoholic solvent with ethanol 34%, 42% and 70% for 48 hours. The solution was filtered through filter paper. Thymol and carvacrol standard materials were purchased from Merck (Germany) and Sigma (Germany) companies.

Analysis

The HPLC system consists of a model K-1001 solvent delivery system equipped with a Rheodyne injection valve (20 µl sample loop inserted) and a UV-Vis spectrophotometer detector model K-2600 set at 274 nm (all from Knauer Assoc., Germany). Analyze was performed by using an ODS-C18 column (150 × 4.6 mm i.d., 5 µm particle size), and the corresponding guard column. All solvent were filtered and degassed earlier entering the column.

For separation of carvacrol (Merck, Germany) peak from thymol (Sigma, USA), different solvents as mobile

phase in HPLC (methanol, acetic acid, water and acetonitrile) were used. Finally the mobile phase was selected an isocratic combination of acetonitrile: H₂O: acetic acid (65:34:1). The mobile phase flow rate was 1.0 ml/min, and all the measurements were done at ambient temperature. In this mobile phase, the peaks of thymol and carvacrol were successfully separated in the samples and pure peak of thymol was reached for quantification. Stock solutions of thymol (0.5 mg/ml) were prepared. Different concentrations (0.025, 0.05, 0.1, 0.25 and 0.5 mg/ml) were prepared from stock solution.

Statistical analysis

Data were presented as mean \pm standard deviation (SD) for three experiments. The results were analyzed by Excel software.

Result

The separation of thymol and carvacrol with reversed phase chromatography was provided with several mobile phases, because these phenols have similar chemical structures (Fig. 1). First we selected methanol-water mobile phase, however, thymol and carvacrol were not completely separated and with tailing in their chromatogram peaks. The best mobile phase was selected by varying proportion acetonitrile by adding acetic acid. Thymol and carvacrol was separated by best mobile phase of acetonitrile: H₂O: acetic acid (65:34:1) at isocratic elution. In ZM extract, thymol and carvacrol were showed a retention times 10.4 and 9.8 minutes, respectively (Fig. 2). For finding of each peaks is on thymol or cravacrol, we injected thymol and carvacrol standards also addition of these standards to extract for

adjustments of these phenols in Zatraia extract (Fig. 3).

Calibration graph was performed using the external standard technique following linear regression analysis by plotting concentration against peak area. Figure 3 shows the equations got for the calibration graphs and the regression coefficients ($r^2 = 0.998$) (Fig 4). Samples of ZM extract (70, 42 and 34% hydroalcoholic extract) were injected three times and chromatographs were reached. These samples were quantified from line equation of calibration curve, which was got from thymol standards. Table 1 showed the thymol in each hydroalcoholic extracted samples. Thymol content was 2.7 ± 0.06 , 3.7 ± 0.07 and 6.0 ± 0.11 mg/ g dried extract for ethanol concentration of 34, 42 and 70%, respectively.

Discussion

Z. multiflora has several biological and pharmacological properties. ZM is acted as an antimicrobial in the food industry (14-17) and used as an antispasmodic, anesthetic agent in Iranian traditional medicine (14). There are many studies that show that *Z. multiflora* has antibacterial (9) antifungal and antioxidant (7, 8) activities. Thymol acts on the microbial cell membrane and causes substantial morphological damage, resulting in a change in permeability and the release of cellular contents (18,19). In previous studies, GC-MS instrument was performed for analysis the essential oil of ZM. The content of thymol in *Zataria* hydro alcoholic extract was analyzed by HPLC method, but the carvacrol peak was not identified in chromatography method (13). ZM is used as an important herbal medicine in Iranian traditional medicine for 1000

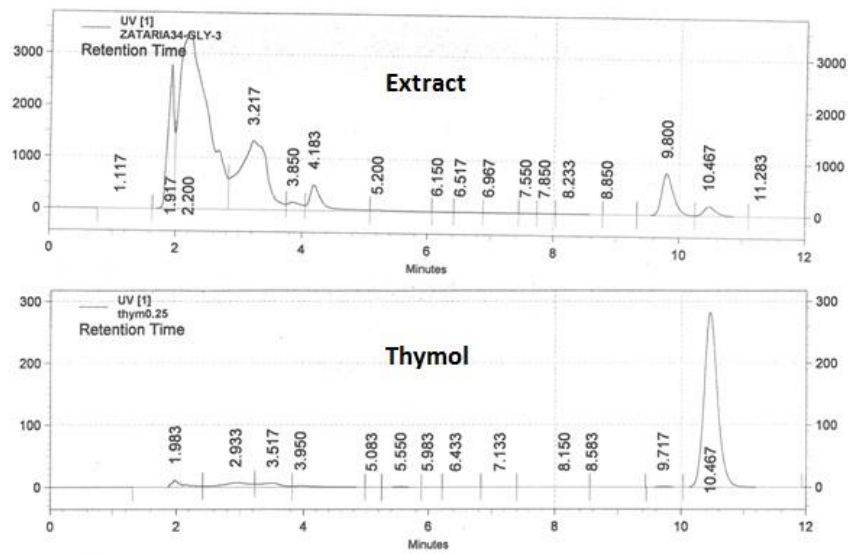


Figure 2 HPLC chromatograms of *Zataria multiflora* extract and thymol analyses with a mobile phase of acetonitrile: H₂O: acetic acid (65:34:1).

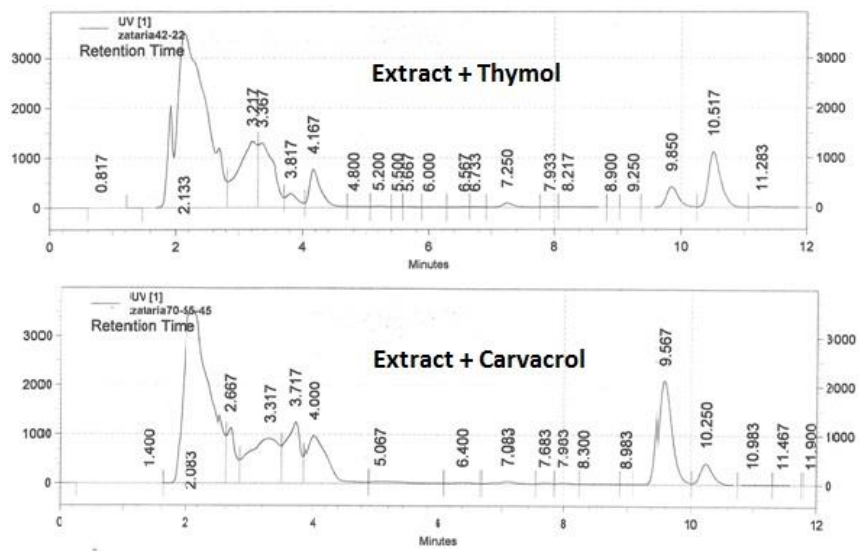


Figure 3 HPLC chromatograms of *Zataria multiflora* extract + thymol and *Zataria multiflora* extract + carvacrol that analyses with a mobile phase of acetonitrile: H₂O: acetic acid (65:34:1)

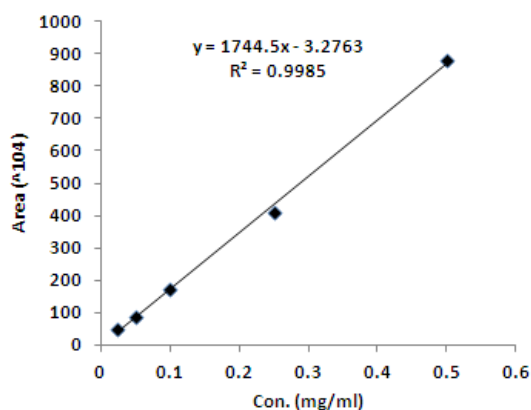


Figure 4 Thymol calibration curve

Table 1 Thymol content in different hydroalcoholic extract of *Zataria multiflora* extracted with different proportion of ethanol.

Percentage of ethanol for extraction	Thymol mg/g extract (mean \pm SD)
70	6.0 \pm 0.11
42	3.7 \pm 0.07
34	2.7 \pm 0.06

years to cure stomachache and agitation, and to combat insect bites (Canon of Medicine) (10) ZM can be natural therapeutic agents for the treatment of Alzheimer's disease. Antioxidant, anti-inflammatory, and anticholinesterase properties of thymol might contribute to its beneficial effects (20). Kavosi showed that ZM mitigated oxidative stress and may be used in the therapy of oxidative damage accompanying hyperglycemia and some inflammatory conditions (8). Thymol and carvacrol are isomeric non-polar phenolic compounds that are only slightly soluble in water at neutral pH, but they are extremely soluble in alcohols and other organic solvents. As results showed in figure 2, thymol has retention time about 1 minute more than carvacrol, it is

showed thymol is more non-polar than carvacrol in chemical structure. Thymol and carvacrol have similar chemical structure with a difference in position of hydroxyl group (Fig. 1). Hydroxyl group is close to short chain of isopropyl for thymol, while this group is close to methyl group for carvacrol. The positions of hydroxyl group affect on polarity of these phenols. One of the aims of our study was the effects of percentage of ethanol on the extraction of thymol from ZM herb, because thymol is the most phenolic compound in this herb for pharmacological effects. Thymol (2-isopropyl-5-methylphenol) is found in oil and extract of thyme and plants including *Thymus vulgaris* and ZM. Thymol is soluble in aqueous ethanol, and highly soluble in non-polar organic solvents, and the result of our study showed that the thymol content increases in Z. multiflora extract by increasing ethanol proportion. It was 2.7 \pm 0.06 and 6.0 \pm 0.11 mg/g in ethanol 34 and 70%, respectively.

Conclusion

In this study, a mobile phase for the separation of thymol and carvacrol as phenolic compounds in ZM by using HPLC method. This method is a useful tool for the assay of thymol in ZM extract as a pharmaceutical dosage form, which is one of the important herbal medicines. We showed that the concentration of thymol in the hydroalcoholic extract of ZM is dependent on ethanol concentration in the extraction solvent.

Conflict of interest

The authors declared no potential conflict of interest with respect to the authorship, and/or publication of this study.

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